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Inhibition of Photo-Induced Electron Transport and Related Reactions in Isolated Chloroplasts by Phenol

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Summary. Phenol inhibits the Hill reaction with several Hill oxidants and the accompanying non-cyclic phosphorylation. It inhibits also pseudocyclic and cyclic phosphorylation. Partial reactions which are dependent on cyclic electron flow are inhibited too, but electron flow from ascorbate via dichlorophenolindophenol to TPN is not. The activity of various benzene and phenol derivatives was compared. It is concluded that the inhibition is a result of interfering with an electron carrier which participates in cyclic and non-cyclic electron flow.

The use of inhibitors is a potent tool in the elucidation of the mechanism of electron transport and the accompanying phosphorylation in isolated chloroplasts. Most of the known inhibitors of electron transport presumably inhibit some reaction near the oxygen evolution site (8). The evidence for this includes the finding that while non-cyclic electron flow and the accompanying phosphorylation are inhibited, cyclic phosphorylation is not.

Indiscriminating inhibition of phosphorylation is caused by uncouplers, but few compounds are known which inhibit the 2 types of phosphorylation by inhibiting a common part of electron transport. The data presented indicate such activity for phenol and various phenol derivatives.

Materials and Methods

Chloroplasts were prepared from lettuce (*Lactuca sativa* var. romaine). Thirty grams of leaves were blended in a 220 V Waring blendor for 20 seconds at 120 V, in 70 ml of medium containing 0.4 M sucrose, 0.01 M NaCl, 0.05 M tris, 0.05 M ascorbate (pH 8.0). The homogenate was filtered through gauze and centrifuged at low speed. The chloroplast pellet was then collected by centrifuging for 7 minutes at 1500 × *g*. The chloroplasts were washed once in the homogenizing medium or in the same medium without ascorbate, when ferricyanide reduction was measured, centrifuged again for 7 minutes at 1500 × *g* and resuspended in the original medium (without ascorbate) at a concentration of approximately 1 mg chlorophyll/ml.

Chlorophyll was determined according to the procedure of Arnon (1). Ferricyanide reduction was measured by loss of absorption at 420 mμ of the deproteinized solution. DCPIP[1] reduction was followed by measuring the decrease in OD at 620 mμ in an Uvispec spectrophotometer or at 570 mμ by continuous recording in a Cary 15 spectrophotometer. In the latter the actinic beam was provided by a 300-W slide projector passed through a red filter (Corning No. 2403) at right angles to the measuring beam. The phototube was protected from the actinic beam by a blue filter (Corning filter No. 9782). TPN reduction was followed by measuring OD increase at 340 mμ. Ferredoxin was prepared from mangold leaves according to the procedure of Hill and Bendall (10), up to and including the column fractionation on DEAE-cellulose. Phosphorylation was measured by following ^{32}P incorporation into ATP according to Avron (3). Light induced proton uptake was measured by titration with a TTT-1 Titrigraph as reported (17). Light scattering was measured by recording OD at 520 mμ with a Cary 15 spectrophotometer using the same illumination and filter arrangement as that for DCPIP reduction. The light induced ATP-ase was measured as described by Petrack et al. (19).

Results

The effect of phenol on ferricyanide reduction and the accompanying phosphorylation is shown in table I. The reduction is inhibited whether the phosphorylating reagents are present or not, indicating that the inhibition of ATP formation is a result of electron flow inhibition. The results of table I clearly demonstrate that cyclic ATP synthesis with PMS is inhibited as much as the Hill reaction with ferricyanide. Phosphorylation mediated by other carriers (table II) is also inhibited.

[1] Abbreviations: PMS, phenazine methosulphate; DCPIP, 2,6-dichlorophenolindophenol; BDHB, *n*-butyl-3,5-diiodo-4-hydroxybenzoate; DCMU, 3-(3,4-dichlorophenyl)-1,1 dimethylurea.

Table I. *Effect of Phenol on Ferricyanide Reduction and Ferricyanide and PMS Phosphorylation*

The reaction mixture for PMS phosphorylation contained in 3.0 ml the following in μmoles: tris 50 (pH 7.8); NaCl 120; $MgCl_2$ 20; Pi 10; ADP 4; PMS 0.15; 6×10^5 cpm of ^{32}P and chloroplasts containing 60 μg chlorophyll. The reaction mixture for ferricyanide dependent phosphorylation was the same but PMS was replaced by 1.8 μmoles of the former.

Illumination was from 150 Watt bulb shielded by a water bath providing 3500 ft-c at the level of the test tubes. The reaction with PMS was run for 5 minutes and with ferricyanide for 3 minutes it was terminated by turning off the light and adding 0.3 ml of 30 % trichloroacetic acid.

Control values: ferricyanide reduction = 368 μmoles per hour per mg chlorophyll; in the presence of phosphorylating reagents S.A. = 455. Ferricyanide mediated phosphorylation — 158 μmoles of ATP formed per hour per mg chlorophyll. PMS phosphorylation — 275 μmoles of ATP formed per hour per mg chlorophyll.

Phenol concentration	ACTIVITY Ferricyanide Reduction*	Ferricyanide Reduction	Ferricyanide ATP formation	PMS ATP formation
M	%	%	%	%
0	100	100	100	100
5×10^{-3}	72	66	40	62
10^{-2}	58	42	14	37
2×10^{-2}	24	17	0	3

* No phosphorylating reagents.

Table II. *Inhibition of Phosphorylation in the Presence of Various Cofactors by Phenols*

Phenol was added at 10 mM. Otherwise experimental conditions as in table I.

Cofactor	ATP Formation No Phenol	ATP Formation Plus Phenol
	μmoles/hr mg chlor	% of control
PMS, 0.05 mM	339	35
Pyocyanine, 0.05 mM	340	21
Vitamin K_5, 0.05 mM	171	10
FMN, 0.05 mM	185	7

Table III. *Effect of Phenol on Electron-Transport*

The control value of ferricyanide reduction (S.A.) in the atebrin experiment was 272; with atebrin 570; in NH_4Cl experiment 236; with NH_4Cl 465; otherwise experimental conditions of ferricyanide reduction as in table I.

The reaction mixture for TPN reduction contained in a total volume of 1.5 ml the following in μmoles: tris 25 (pH 7.8); NaCl 60; TPN 0.25. Saturating amounts of ferredoxin and chloroplasts containing 30 μg chlorophyll. When ascorbate was the electron donor there were also in μmoles: ascorbate 7.5; DCPIP 0.075 and DCMU 0.015; illumination as described in table I. Specific activity of TPN reduction from H_2O – 74; from ascorbate DCPIP – 50.

DCPIP was measured in bean chloroplasts which were isolated at pH 6.0 according to McCarty and Jagendorf (16). The reaction mixture contained in a total volume of 10 ml the following in μmoles: sodium citrate 200 (pH 4.5); NaCl 80; DCPIP 0.3 and chloroplasts containing 22 μg chlorophyll. Reduction was measured by a potentiometric titration with ferricyanide (16). The control value of DCPIP reduction was 1440 μequivalents per hour per mg chlorophyll.

Phenol Concn	ACTIVITY Ferricyanide reduction Control	+Atebrin 0.1 mM	Control	+NH_4Cl 1 mM	DCPIP Reduction	TPN reduction H_2O	Ascorbate DCPIP
M	%	%	%	%	%	%	%
	100	100	100	100	100	100	100
3.2×10^{-3}	68	60	90	90	...	66	108
6.5×10^{-3}	47	20	76	27	42	37	112
1.3×10^{-2}	26	12	57	21	25	12	102
2.6×10^{-2}	15	9	27	18	0	9	101

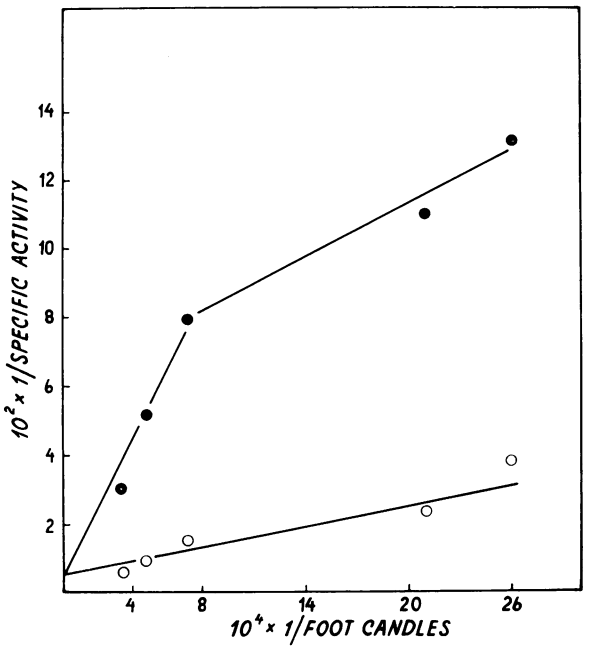

FIG. 1. Rate of DCPIP reduction as a function of light intensity. Light intensity is shown as reciprocal of the percentage transmission of various filters used. The intensity with no filter, before passing through the red filter, was approximately 30,000 ft-c. The reaction mixture contained in 3.0 ml the following in μmoles: tris 50 (pH 7.8); NaCl 120; DCPIP 0.12; chloroplasts equivalent to 20 μg chlorophyll. ○ – Control; ● – 20 mM phenol. The dye reduction was followed with a Cary spectrophotometer.

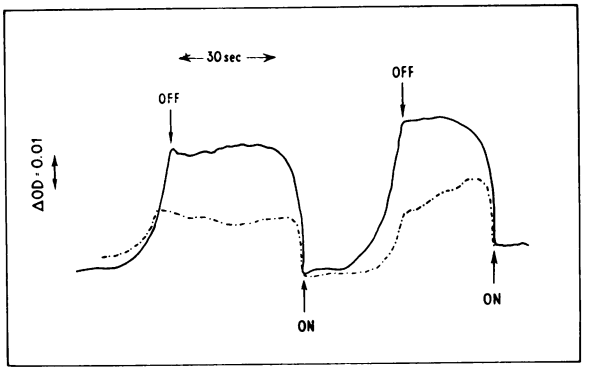

FIG. 2. The effect of phenol on the photoinduced change in light scattering. The reaction mixture contained in a total volume of 3 ml the following in μmoles: Na-citrate 100, (pH 6.3); NaCl 120; pyocyanine 0.15 and chloroplasts equivalent to 40 μg chlorophyll. Solid lines control. Dashed lines 13 mM phenol. The absorbency change was followed with a Cary spectrophotometer.

Phenol inhibits electron transport which is uncoupled either by atebrin or ammonium chloride as shown in table III. Actually the stimulated electron flow is inhibited somewhat more than the basal rate.

Bean chloroplasts isolated at pH 6.0 contain presumably only a part of an active electron transport pathway which can reduce DCPIP at low pH (16). The results of table III show that phenol inhibits this residual electron transport.

TPN reduction is inhibited by phenol when water serves as the electron donor, but reduction by ascorbate DCPIP is not inhibited (table III). The reduction of TPN by ascorbate was found to be dependent on ferredoxin even in the presence of phenol.

Figure 1 presents the degree of inhibition of DCPIP reduction by phenol as a function of light intensity in a double reciprocal plot (1/velocity vs. 1/light intensity). The most pronounced effect is on the light reaction.

Partial reactions in the presence of PMS or pyocyanine which presumably depend on cyclic electron flow, namely light-induced proton uptake and light-induced absorbancy change are strongly inhibited by phenol (table IV and fig 2). The same is true of the thiol-dependent ATP-ase discovered by Petrack et al. (19) as shown in table V.

When TPN reduction from ascorbate, DCPIP and cyclic phosphorylation mediated by this couple are performed simultaneously the latter is strongly inhibited whereas the former is not. It is occasionally stimulated (table VI).

On testing the chemical specificity of inhibition it was found that benzene does not inhibit cyclic phosphorylation and among the benzene derivatives only aniline and chlorobenzene in addition to phenol are inhibitory (table VII). Substitution in the benzene ring ortho to phenol, by either hydroxyl, aldehyde or aldoxime increases appreciably the inhibitory activity of the compound (table VII).

Discussion

Phenol was shown to inhibit electron transport with 3 different Hill oxidants: TPN, DCPIP and ferricyanide (table III). As expected ATP formation accompanying the latter is inhibited too (table I). However, at variance with compounds like

Table IV. *Effect of Phenol on Light-induced Proton Uptake in Isolated Chloroplasts*

Fragmented chloroplasts were prepared in 10 mM NaCl as reported (17). The reaction mixture contained in 8 ml, chloroplasts equivalent to 30 μg of chlorophyll, PMS 0.1 mM and NaCl 100 mM. The extent of proton uptake was measured by titration with acid at pH 6.2. The control value was 0.3 μequivalent H^+ taken up per mg chlorophyll. Illumination conditions as in table I.

Phenol concn	Proton uptake
M	% of activity
0	100
3×10^{-3}	80
1.3×10^{-2}	70
2.6×10^{-2}	45

Table V. *Effect of Phenol on Light-induced ATP-ase*

The reaction mixture contained in a total of 3.05 ml the following in μmoles: tris 50 (pH 7.8); NaCl 120; $MgCl_2$ 20; PMS 0.15; cysteine 180; ATP 5; and chloroplasts containing 150 μg chlorophyll. The reaction mixture was illuminated for 2 minutes (light intensity 8000 ft-c) and was transferred to the dark for additional 13 minutes. It was stopped by 0.3 ml trichloroacetic acid. Pi release was tested according to the procedure of Carmeli and Avron (7). The control value for ATP-ase activity was 49 μmoles ATP hydrolyzed per mg chlorophyll per hour.

Phenol concn	ATP-ase Activity	
	Phenol in light	Phenol in dark
M	% of Control	
10^{-2}	59	100
2×10^{-2}	0	40

Table VI. *Effect of Phenol on Cyclic Phosphorylation with Ascorbate and DCPIP and on TPN Reduction with Ascorbate + DCPIP*

The reaction mixture contained in a total volume of 1.5 ml the following in μmoles: tris 25 (pH 7.8); NaCl 60; ascorbate 7.5 DCPIP 0.075 and DCMU 0.015, $MgCl_2$ 10; Pi 5; ADP 2; 6×10^5 cpm of ^{32}P and chloroplasts containing 30 μg chlorophyll when cyclic phosphorylation was measured in the —TPN series. In the +TPN series the reaction mixture as above but 0.25 μmoles of TPN and saturating amounts of ferredoxin were added.

	TPN Reduction	ATP Formation	
		+TPN	—TPN
	μmoles/hr mg chlor	μmoles/hr mg chlor	
Control	10	76	73
+Phenol, 13 mM	17	18	4

substituted phenylureas (8), atrazine (9), hydroxyquinoline oxides (4) and BDHB (6), which supposedly inhibit close to the oxygen evolution site, the same concentration of phenol inhibited likewise cyclic phosphorylation (tables I and II).

The reduction of DCPIP at low pH by damaged bean chloroplasts seems to be carried out by a shortened segment of electron transport, perhaps by system II only (16). The carrier affected by phenol also participates in this system (table III). Assuming a common site for inhibition of the Hill reaction and cyclic phosphorylation, the most probable would be an electron transport carrier which participates in both reactions. Contrary to previous claims (12) reduction of TPN by the ascorbate-DCPIP couple is probably not accompanied by ATP formation (5). This is supported by the data of table VI. Phenol inhibited cyclic phosphorylation with ascorbate-DCPIP while stimulating TPN reduction from ascorbate. Actually the formation of ATP and its inhibition by phenol are not affected by the presence of TPN. If it is assumed that $DCPIPH_2$ donates the electrons at an identical site in cyclic phosphorylation and in TPN reduction, it is hard to visualize, according to the currently accepted series formulation, an electron transport carrier prior to $DCPIPH_2$ entrance which is common to non-cyclic and cyclic phosphorylation. It is possible that in cyclic phosphorylation the site of donation of electrons by $DCPIPH_2$ is closer to system II than in TPN reduction, as suggested by Trebst and Pistorius (20). Another possibility is that the carriers of cyclic phosphorylation do not act by bridging a potential gap in the photosynthetic electron chain, but by imposing a right redox balance which starts an endogenous cycle of electron flow, in which the phenol inhibited carrier participates.

Plastoquinone is known to be required for the Hill reaction and for cyclic phosphorylation (14), but not when TPN is reduced by ascorbate (2), therefore it might be the carrier inhibited by phenol. However, the fact that phenol inhibits the light-induced ATP-ase (table V) is contrary to the finding in *Anabena variabilis* (15).

The substituted phenol salicylaldoxime was found by Trebst et al. (21) to inhibit both the Hill reaction and phosphorylation, which he ascribed to the

Table VII. *Inhibition of Cyclic Phosphorylation by Benzene Derivatives and Substituted Phenols*

PMS served as cofactor for cyclic phosphorylation. Otherwise experimental conditions as in table I.

Addition	ATP Formation			
	Compound added at 10 mM		Compound added at 5 mM	
	μmoles/hr mg chlor	% of Control	μmoles/hr mg chlor	% of Control
None	331	100		
Benzene	326	98		
Benzoic acid	293	88		
Aniline	285	81		
Chlorbenzene	244	74		
Phenol	196	59	323	79
None	380	100	405	100
Salicylic acid	360	95	...	...
O-Cresol	39	10	177	43
Salicyl aldehyde	11	3	265	65
Salicyl aldoxime	11	3	188	46

binding of copper in plastocyanine. Since several ortho-substituted phenols, and phenol are also inhibitors (table VII), their action cannot be due to chelation of copper. This is also unlikely in view of the fact that both salicyl-aldoxime (21) and phenol do not inhibit TPN reduction from ascorbate, but plastocyanine is thought to participate in the latter reaction (11, 23).

Dinitrophenol is known to be both an electron transport inhibitor (13) and an uncoupler (18).

The phenol inhibition cannot be due to a non-specific effect on proteins, since reduction of TPN by ascorbate-DCPIP is not affected. Although it has been shown recently (22) that this reaction can be catalyzed by chlorophyllin with the participation of TPN reductase only, in chloroplast particles, however, at least ferredoxin (24) and plastocyanine (11) are required as well. In addition the chloroplast reaction as contrasted with that catalyzed by chlorophyllin, depends on the presence of DCPIP. An absolute dependence on ferredoxin and to a large degree on DCPIP was shown in the presence of phenol.

Finally, it cannot be entirely ruled out that phenol and phenol derivatives act as energy transfer inhibitors close to the electron transport chain. This possibility is indicated to a certain extent by the higher susceptibility to phenol inhibition when phosphorylating reagents are present (table I) and by the fact that some inhibition of the ATP-ase takes place even during the dark stage (table V).

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Literature Cited

1. Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24: 1–15.
2. Arnon, D. I. and A. A. Horton. 1963. Site of action in plastoquinone in the electron transport chain of photosynthesis. Acta Chem. Scand. 17: S135–39.
3. Avron, M. 1960. Photophosphorylation by Swiss chard chloroplasts. Biochim. Biophys. Acta 40: 257–72.
4. Avron, M. 1961. The inhibition of photoreactions of chloroplasts by 2-alkyl-4-hydroxyquinoline n-oxides. Biochem. J. 78: 735–39.
5. Avron, M. 1964. On the site of ATP formation in phosphorylation. Biochem. Biophys. Res. Commun. 17: 430–32.
6. Avron, M. and N. Shavit. 1965. Inhibitors and uncouplers of photophosphorylation. Biochim. Biophys. Acta 109: 317–31.
7. Carmeli, C. and M. Avron. 1966. A light-triggered ATP-Pi exchange activity in chloroplasts. Biochem. Biophys. Res. Commun. 24: 923–28.
8. Jagendorf, A. T. 1962. Biochemistry of energy transformations during photosynthesis. In: Survey of Biological Progress, Vol. IV. Academic Press, New York. p 181–344.
9. Good, N. E. and I. Seikichi. 1965. Selective inhibitors of photosynthesis. In: Biochemical Dimensions of Photosynthesis. D. W. Krogmann and W. H. Powers, eds. Wayne State University Press, Detroit, Michigan.
10. Hill, R. and F. Bendall. 1960. Crystallization of a photosynthetic reductase from a green plant. Nature 187: 147.
11. Kato, S. and A. Takamiya. 1965. Restoration of NADP photoreduction activity of sonicated chloroplasts by plastocyanin. Biochim. Biophys. Acta 99: 156–60.
12. Losada, M., F. R. Whatley, and D. I. Arnon. 1961. Separation of two light reactions in noncyclic photophosphorylation of green plants. Nature 190: 606–10.
13. Krogmann, D. W., A. T. Jagendorf, and M. Avron. 1959. Uncouplers of spinach chloroplasts photosynthetic phosphorylation. Plant Physiol. 34: 272–77.
14. Krogmann, D. W. and E. Olivero. 1962. The specificity of plastoquinone as a cofactor for photophosphorylation. J. Biol. Chem. 237: 3292–95.
15. Lightbody, J. J. and D. W. Krogmann. 1966. The role of plastoquinone in the photosynthetic reactions of *Anabaena variabilis*. Biochim. Biophys. Acta 120: 57–64.
16. McCarty, R. E. and A. T. Jagendorf. 1965. Chloroplast damage due to enzymatic hydrolysis of endogenous lipids. Plant Physiol. 40: 725–35.
17. Neumann, J. and A. T. Jagendorf. 1964. Light-induced pH changes related to phosphorylation by chloroplasts. Arch. Biochem. Biophys. 107: 109–19.
18. Neumann, J. and A. T. Jagendorf. 1964. Dinitrophenol as an uncoupler of photosynthetic phosphorylation. Biochem. Biophys. Res. Commun. 16: 562–67.
19. Petrack, B., A. Creston, F. Sheppy, and F. Farron. 1965. Studies on the hydrolysis of adenosine triphosphate by spinach chloroplasts. J. Biol. Chem. 240: 906–14.
20. Trebst, A. and E. Pistorius. 1965. Zum Mechanismus des photosynthetischen Electronentransportes in isolierten Chloroplasten. Z. Naturforsch. 20b: 143–47.
21. Trebst, A., H. Eck, and S. Wagner. 1963. Effects of quinones and oxygen in the electron transport system of chloroplasts. In: Photosynthetic Mechanisms of Green Plants. Kok B. and A. T. Jagendorf, eds. National Academy of Sciences, National Research Council, Washington, DC, U.S.A. p 174.
22. Vernon, L. P., A. San-Pietro, and D. Limbach. 1965. Photoreduction of NAD and NADP by chlorophyllin in the presence of ascorbate: requirement for NADP reductase. Arch. Biochem. Biophys. 109: 92–97.
23. Vernon, L. P. and E. Shaw. 1965. Photochemical activities of spinach chloroplasts following treatment with the detergent triton X-100. Plant Physiol. 40: 1269–77.
24. Vernon, L. P. and W. S. Zaugg. 1960. Photoreductions by fresh and aged chloroplasts: requirement for ascorbate and 2,6-dichlorophenolindophenol with aged chloroplasts. J. Biol. Chem. 235: 2728–33.